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Patterning of the Avian Intermediate Mesoderm by Lateral Plate and Axial Tissues

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Amniote kidney tissue is derived from the intermediate mesoderm (IM), a strip of mesoderm that lies between the somites and the lateral plate. While much has been learned concerning the later events which regulate the differentiation of IM into tubules and other types of kidney tissue, much less is known concerning the earlier events which regulate formation of the IM itself. In the current study, the chick pronephros was used as a model system to identify tissues that play a role in patterning the IM and the critical time periods during which such patterning events take place. Explant studies revealed that the prospective pronephric IM is already specified to express kidney genes by stage 6, shortly after its gastrulation through the primitive streak, and earlier than previously reported. Transplant and explant experiments revealed that the lateral plate contains an activity that can repress IM formation in tissues that are already specified to express IM genes. In contrast, Hensen's node can promote formation of IM in the lateral plate. Paraxial tissues (presomitic mesoderm plus neural plate and notochord) were found to influence the morphogenesis of the nephric duct, but did not induce IM tissue to an appreciable extent. Combining lateral plate and paraxial tissue *in vivo* or *in vitro* led to induction of IM genes in the paraxial mesoderm but not in the lateral plate mesoderm. Based on these results and those of others, we propose a two-step model for the patterning of the IM. While tissue is still in the primitive streak, the prospective IM is relatively uncommitted. By stage 6, shortly after cells leave the primitive streak, a field of cells is generate which is specified to give rise to IM (Step 1). Subsequently, competing signals from the lateral plate and axial tissues modulate the number of cells that commit to an IM fate (Step 2).

Key Words: **pronephros; kidney; chick embryo; cell fate determination; fate map.**

INTRODUCTION

The major functional units of the vertebrate nephric system are the glomerulus, which filters the blood; the tubule, where absorption and secretion of metabolites takes place; and the nephric duct, which drains the tubules. All components of the nephric system are derived from the intermediate mesoderm (IM), a strip of mesoderm that lies between the somites and the lateral plate (LP) (Fig. 1) (Saxen, 1987). In amniotes, the nephric duct (also called the Wolffian duct or the pronephric duct) forms from the anterior-most region of the IM (in chick, the IM adjacent to somites 6-10) (Obara-Ishihara *et al.*, 1999). During development, this duct rudiment migrates posteriorly and assumes a position dorsal to the remaining IM (which is termed the nephrogenic cord) (Saxen, 1987). The nephrogenic cord gives rise to tubule and glomerular tissue.

From anterior to posterior, the IM differentiates into three types of nephric tissue: the pronephros, the mesonephros, and the metanephros. A large number of studies have investigated the formation of the mammalian metanephros and have found that the metanephros forms as the result of reciprocal inductive interactions between a branch from the nephric duct (the ureteric bud) and the posterior nephrogenic cord (the metanephric mesenchyme) (Sariola and Sainio, 1997). However, while several heterologous tissues and molecules can induce tubule formation in the metanephric mesenchyme (Lombard and Grobstein, 1969; Unsworth and Grobstein, 1970; Herzlinger *et al.*, 1994), IM mesenchyme is the only known tissue that is competent to produce tubules in response to interaction with the ureteric bud. Therefore, it is apparent that the IM has undergone a significant degree of patterning prior to its interaction with ureteric bud *in vivo*.

In order to investigate these earlier events of IM patterning, researchers have turned to studying the development of the first IM derivatives to form: the nephric duct rudiment and the pronephros (Vize *et al.*, 1997; Mauch *et al.*, 2000).

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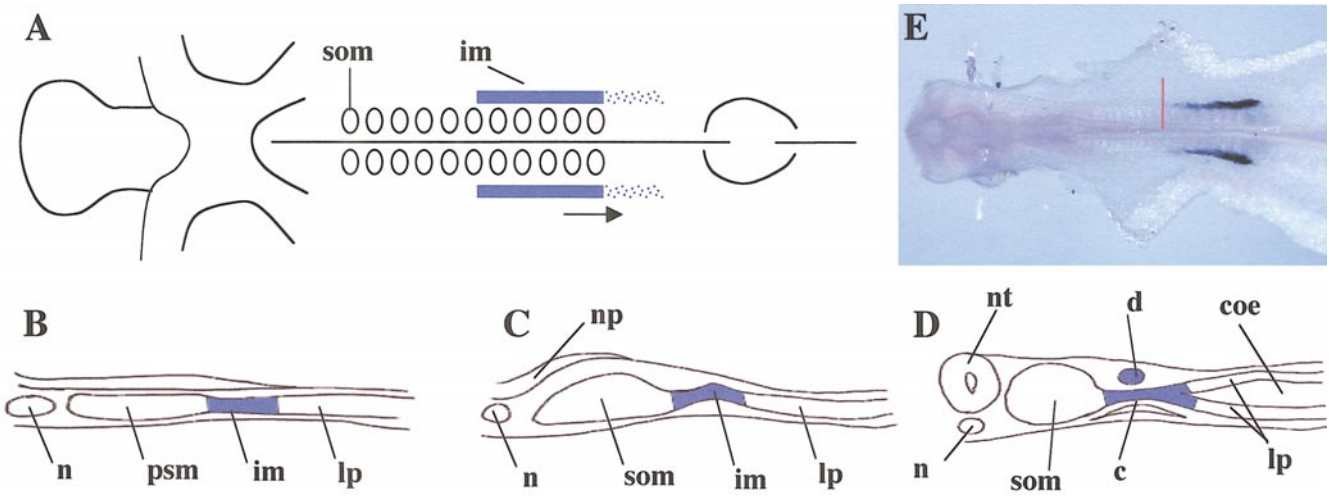


FIG. 1. Diagram of early chick pronephros morphogenesis. (A) Whole-mount view illustrating that the kidney tissue forms from the intermediate mesoderm (blue) located adjacent to the somites and that formation of kidney structures proceeds in an anterior to posterior sequence. (B–D) Diagrams of sections through stage 8 (B), stage 9 (C), and stage 11 (D) pronephros, illustrating the morphogenetic movements that the intermediate mesoderm undergoes to the produce the nephric duct and nephrogenic cord. (E) Expression of lim-1 mRNA in stage 11 embryos, illustrating that the anterior border of lim-1 expression is at the axial level of the sixth somite (red line indicates sixth somite). c, nephrogenic cord; coe, coelom; d, nephric duct; im, intermediate mesoderm; lp, lateral plate; n, notochord; np, neural plate; nt, neural tube; psm, presomitic mesoderm; som, somite. In (A) and (E), anterior is on the left.

Several transcription factors have been described which are expressed in the pronephros and are required for normal tubule and/or duct development in mammalian and amphibian species. Lim-1 (Fujii *et al.*, 1994) is expressed in a large region of the *Xenopus* mesoderm shortly after gastrulation and is later restricted to the pronephros (Taira *et al.*, 1994; Carroll and Vize, 1999; Carroll *et al.*, 1999). In the lim-1 knockout mouse, no nephric derivatives are formed, including the pronephric duct, suggesting an important role for lim-1 in kidney development (Shawlot and Behringer, 1995). Pax-2 and the related gene pax-8 are paired domain-containing homeobox proteins which are expressed early during the formation of the IM (Dressler *et al.*, 1990; Krauss *et al.*, 1991; Drummond *et al.*, 1998; Carroll and Vize, 1999). Mice lacking pax-2 have degenerate nephric ducts. Although they form nephrogenic cord and metanephric mesenchyme, these tissues do not undergo tubulogenesis (Torres *et al.*, 1995). These studies suggest that pax-2 is required either in the IM for tubule differentiation or in the duct for induction of tubule differentiation, or both. In *Xenopus*, pax-8 has been shown to be sufficient to induce ectopic tubule development in somitic tissue (Carroll and Vize, 1999).

The embryonic patterning events that lead to expression of these transcription factors in the IM, and to formation of the pronephric duct and tubules, are not well understood. In both amphibian and avian embryos, signals from medial/dorsal structures, including the somites, have been found to promote pronephric gene expression (Seufert *et al.*, 1999; Mauch *et al.*, 2000), and surface ectoderm is required for maintenance of high levels of IM gene expression in the nephric duct (Obara-Ishihara *et al.*, 1999). The role of other embryonic tissues in patterning the pronephros has not been determined. At the molecular level, Bmp, activin, and retinoic acid signaling have been implicated in kidney tubule formation in *in vitro* systems (Green and Smith, 1990; Moriya *et al.*, 1993; Dosch *et al.*, 1997), although the role of these factors in early IM patterning *in vivo* is not clear. At later stages, Notch signaling has been found to regulate duct vs tubule differentiation within the *Xenopus* pronephros (McLaughlin *et al.*, 2000) and BMP signaling has been shown to be required for maintenance of gene expression in the nephric duct (Obara-Ishihara *et al.*, 1999).

As a prerequisite to identifying the molecular events that pattern the pronephros, it is important to have a more complete characterization of the tissue interactions that regulate its formation. The aim of this work is to conduct a systematic investigation of the earliest patterning events which regulate kidney development, using the chick pronephros as an experimental system. A series of transplant and explant experiments was performed in order to identify tissues that play a role in regulating IM gene expression and the critical time periods during which such patterning events take place. These studies indicate that IM formation is regulated by a combination of signals from axial and lateral tissues.

METHODS

Embryo Culture

For *in vivo* fate-mapping and transplantation experiments, chick embryos (White Leghorn; Hy-Line, PA) were incubated by using a

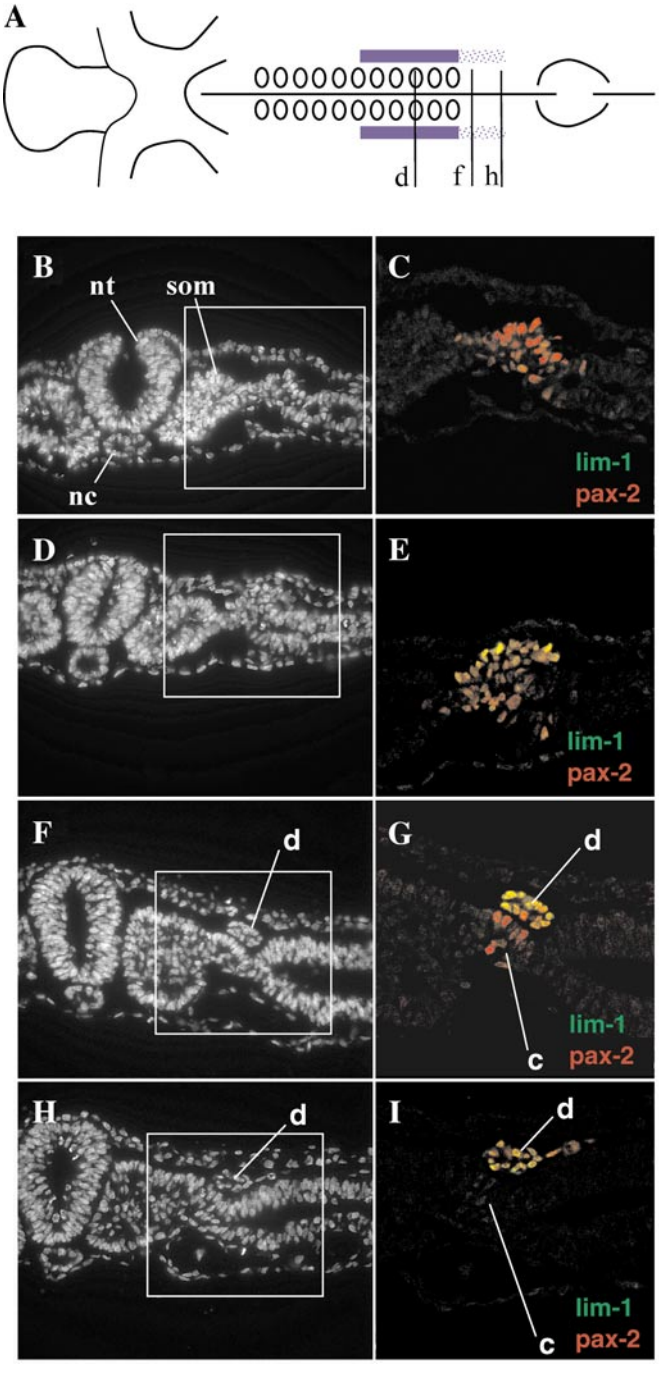


FIG. 2. Dynamics of lim-1 and pax-2 protein expression during early kidney formation. Sections of stage 9 (B, C), stage 10 (D, E), and stage 11 (F–I) embryos, stained with DAPI (B, D, F, H), and antibodies to lim-1/2 (green) and pax-2 (red) (C, E, G, I). Boxes in (B, D, F, H) indicate the area shown at higher magnification in (C, E, G, I). Schematic in (A) lists the axial levels of sections in (D–I). See text for description. c, nephrogenic cord; d, nephric duct; nc, notochord; nt, neural tube; som, somite.

modified form of New culture (New, 1955). Briefly, embryos HH4 and older were collected by attaching a 1-inch diameter paper ring (P5; Fisher) to the vitelline membrane overlying the embryo. The remainder of the vitelline membrane was cut away, and the embryo was washed with 1× Tyrodes buffer and placed endoderm-up on an albumin–agar culture dish (50% albumin, 1.5% glucose, 0.3% agar, 0.9% NaCl) (Sundin and Eichele, 1992). These were incubated at 38°C until the experiment was finished. For explant analysis, chick embryonic tissue was cultured in type I collagen gels (Schultheiss *et al.*, 1997) suffused with defined tissue culture media: DMEM-F12 (Gibco), 5 μg/ml human transferrin (Gibco), 100 μg/ml conalbumin (Sigma), 1× insulin–transferrin–selenium (Gibco), 1% Pen-Strep, and 1% L-glutamine.

Fate Mapping

Embryos were injected with CM-DiI (Molecular Probes) in 0.3 M glucose, using a Picospritzer II injector and Leitz micromanipulator, and were grown in modified New culture. Embryos were photographed for documentation at the beginning of the experiment and at 5-h intervals thereafter, using a Zeiss SV11 dissecting microscope with an epifluorescence attachment, Dage video camera, and Openlab image processing software. After 15 h, some (approximately 20) embryos were grown fixed in 4% paraformaldehyde and cryosectioned to determine the tissue localization of the DiI. Since sectioning revealed that the DiI resided in the mesoderm, as expected, and that the tissue localization of the DiI agreed with the localization as determined by whole-mount epifluorescence detection, the whole-mount data were subsequently used to determine tissue localization.

Transplantation

Tissues were excised from donor quail embryos (*Coturnix coturnix japonica*; Strickland quail farm, GA) by using tungsten needles. For transplants of stages 8 and 9 intermediate mesoderm, somite, and lateral plate, mesoderm alone was removed for grafting. First, two parallel cuts were made on the left and right sides of the tissue of interest. Then, 1 μl of 10 μg/ml Dispase (Gibco/BRL) was pipetted directly onto the area. The mesoderm was separated from the ectoderm and endoderm by using tungsten needles. Donor pieces were cut in an asymmetric manner in order to preserve orientation when placed in the recipient. After labeling the graft with Cell tracker DiI (Molecular Probes), a small transverse cut was made in the meso- and endodermal layers of the recipient. Mesoderm anterior to the cut was removed by sucking with a fine-tipped needle attached to a mouth pipette. Donor pieces were placed into the vacant location in the host mesoderm by using tungsten needles. For experiments involving primitive streak tissue, epiblast and mesendodermal layers were transplanted. The embryos were cultured for 15-18 h, fixed in 4% paraformaldehyde, and stored in PBS until analyzed.

In Situ Hybridization

Whole-mount *in situ* hybridization was performed as described previously (Schultheiss *et al.*, 1995) by using probes to chick lim-1 (Tsuchida *et al.*, 1994) and pax-2 (Burrill *et al.*, 1997; Herbrand *et al.*, 1998). Following development, 20-μm cryostat sections (Leica) were cut on gelatin-embedded embryos, as described previously (Schultheiss *et al.*, 1995). For *in situ* hybridization on explants, tissues were dissected from the collagen gels and then processed as for whole-mount *in situ* hybridization.

Immunocytochemistry

All embryos and explants that were processed for immunocytochemistry were fixed for 20-30 min in 4% paraformaldehyde. These were gelatin-embedded and cryosectioned at 10-μm intervals. The sections were washed in phosphate-buffered Triton solution (0.25% Triton X-100) for 20 min and soaked in blocking solution (1% BSA, 1% goat serum, 1% horse serum, and 0.02% Tween 20 in PBS) for 30 min. The following primary antibodies were used: the monoclonal antibody QCPN (1:5) that recognizes quail cells (Developmental Studies Hybridoma Bank), the monoclonal antibody 4F2 against lim-1 (1:1) (Developmental Studies Hybridoma Bank), and a rabbit polyclonal antibody to pax-2 (1:250) (BAbCo). FITC- and TRITC-coupled secondary antibodies (Jackson Immunoresearch) were used at a 1:250 dilution in PBS–Tween (0.02% Tween 20). To visualize nuclei, the sections were washed once in 1 μg/ml DAPI (Sigma) in PBS–Tween. Images were collected on a Zeiss Axiophot microscope with a SPOT camera or a BioRad MRC1024 confocal microscope. The Developmental Studies Hybridoma Bank antibodies are maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine (Baltimore, MD) and the Department of Biological Sciences, University of Iowa (Iowa City, IA), under Contract N01-HD-2-3144 from NICHD.

RESULTS

Early Expression Patterns of lim-1 and pax-2 in the Embryonic Intermediate Mesoderm and Its Derivatives

The current studies make extensive use of lim-1 and pax-2 as markers of the pronephric IM. While previous studies have documented the expression of these genes during chick kidney formation (Obara-Ishihara *et al.*, 1999; Mauch *et al.*, 2000), interpretation of the current experiments is aided by a more detailed knowledge of the timing of their early expression patterns, which are presented briefly here. Beginning at stage 9$^-$ (6 somites), both lim-1 (Fig. 1E) and pax-2 (data not shown) mRNA are expressed in the mesoderm adjacent to the somites, from the axial level of the 6th somite and posterior (Fig. 1E; lim-1 also has an earlier, broader expression pattern which will be described elsewhere). At stage 9, pax-2 protein is detectable in the IM adjacent to the 6th to 8th somites (Figs. 2B and 2C), while lim-1 protein is first detected at stage 10$^-$ (Figs. 2D and 2E). At stage 10$^-$, when the IM from the 7th to 11th somites begins to migrate dorsally, indicating the onset of duct morphogenesis (Figs. 1A and 1C), pax-2 and lim-1 proteins are coexpressed in most IM cells (Figs. 2D and 2E). Since coexpression of lim-1 and pax-2 is characteristic of the nephric duct (Figs. 2G and 2I), it is likely that these stage 10$^-$, pax-2$^+$/lim-1$^+$ cells will contribute to the nephric duct. After the onset of duct migration at stage 10$^+$ (Fig. 1A), the pax-2$^+$/lim-1$^+$ duct rudiment comes to lie dorsal to the nephrogenic cord (Fig. 1D). Posterior sections of stage 11 IM show that, immediately after it is exposed to migrating primordial duct cells, the nephrogenic cord expresses neither pax-2 nor lim-1 (Figs. 2H and 2I). However, in a slightly more anterior section in which the cord has been in the presence of preduct cells for a longer time period, expression of pax-2 can be detected in several cells of the nephrogenic cord (Figs. 2F and 2G). This sequence of events is repeated as the duct continues to migrate posteriorly. This expression profile suggests that the pax-2$^+$/lim-1$^+$ cells at axial levels 6-10 contribute primarily to the nephric duct and is consistent with the hypothesis that, once the duct begins to migrate over the nephrogenic cord, it induces pax-2 expression in the underlying cord (see Discussion).

TABLE 1
Location of Prospective IM in the Primitive Streak

	LP	IM	SOM	N
<601	21	48	79	48
601–800	79	79	26	53
>800	100	45	0	21

Note. Embryos were injected with DiI in the primitive streak at stages 4–8, and the fate of injected cells was assessed after overnight culture. Rows list distance of the injection site from Hensen's node, in microns. Columns give the percentage of injections that contributed to the given tissue. If a given injection labeled more than one tissue, then each tissue was counted in the table. lp, lateral plate; im, intermediate mesoderm; som, somite; n, number of injections.

Fate Map of the Chick Embryonic Kidney

The trunk mesoderm begins to gastrulate at stage 4 (Rosenquist, 1966; Schoenwolf *et al.*, 1992; Psychoyos and Stern, 1996). Pax-2 and lim-1 are not expressed and/or maintained in pronephric cells until stage 9 (Fig. 2), by which time previous studies have suggested that the pronephros is already largely committed (Mauch *et al.*, 2000). In order to identify tissues which might pattern the embryonic kidney, it is necessary to know the location between stages 4 and 9 of the cells that will give rise to the nephric duct and nephrogenic cord. To generate such a fate map, a lipophilic fluorescent dye was injected into the primitive streak of stage 4-8 embryos and tracked up to 20 h after the initial injection (Fig. 3). Several insights have emerged from this fate map.

First, the location of the prospective kidney relative to other prospective mesodermal tissues within the primitive streak has been determined. As shown in Table 1, the center of the prospective IM lies between 600 and 800 microns from Hensen's node (approximately 30-40% of streak length). As expected, labeling anterior (300-600 microns from node) or posterior (800-1100 microns) primitive streak preferentially labels somite and lateral plate, respectively (Table 1). Many injections labeled more than one tissue, indicating that, at this time, the primordia of the somite, IM, and LP are not well separated. Similar results were obtained for embryos that were injected at any stage between stages 4 and 8, indicating that, between these stages, the position of the prospective IM within the primi-

TABLE 2

Correlation between Timing of Gastrulation and Final Anterior–Posterior Position of Mesodermal Cells

	3/4	5	6	7/8
S1–5 LP	14	0	0	0
S1–5 IM	22	0	0	0
S1–5 SOM	20	0	0	0
S6–10 LP	42	15	0	0
S6–10 IM	38	30	0	0
S6–10 SOM	14	38	6	0
PS10 LP	14	40	50	47
PS10 IM	8	25	50	60
PS10 SOM	0	10	44	53
N	50	40	16	15

Note. Embryos were injected with DiI in the primitive streak, and the fate of injected cells was assessed after overnight culture. Columns list stage of the embryo at the time of injection. Rows list the percentage of injections that ended up at the indicated location. In the row labels, "S1–5 LP" indicates "lateral plate at the axial level of somites 1–5", etc. ps10, presegmental plate posterior to the most posterior somite (10th) somite. Abbreviations as in Table 1.

tive streak is relatively constant. These results are consistent with previous fate maps of the avian primitive streak (Garcia-Martinez and Schoenwolf, 1992; Psychoyos and Stern, 1996).

Second, the data revealed the relationship between the time of gastrulation and the final location of the IM along the anterior–posterior (A-P) axis. Previous lipophilic dye-labeling experiments have demonstrated that the cells comprising the Wolffian duct are largely derived from IM adjacent to somites 7-11 (Obara-Ishihara *et al.*, 1999). The duct-forming IM was labeled by injections into stage 4 and stage 5 primitive streaks (Table 2). Injections into the IM region of the primitive streak after stage 5 labeled cells posterior to somite 11, which are thought to contribute exclusively to nephrogenic cord. All the sections examined in which the anterior border of DiI expression was found posterior to s11 lacked label in the duct (data not shown), which is consistent with this hypothesis.

Third, the migration path of the prospective IM was traced after it left the primitive streak. Knowledge of the migration path allows one to conduct transplantation experiments using nephric precursors at various points in their developmental history (see next section) and allows one to generate hypotheses as to which tissues might be patterning the IM. Embryos were photographed at several time points after DiI injection (Figs. 3A–3D and 3E–3H) and some were sectioned (Figs. 3I and 3J; and data not shown). The cells that form the duct primordia (IM adjacent to somites 7-11) leave the streak starting at stages 4/5 (Table 1; Figs. 3A and 3E). At stage 6-7, the duct precursors are located immediately ventral to the neural plate/nonneural ectodermal border and lateral to Hensen's node (Figs. 3B, 3C, 3F, and 3G). By stage 8, these cells are located at the border between presomitic and lateral plate mesoderm. After stage 9, prospective IM is immediately lateral to the somite (Figs. 3D and 3H). Any or all of these structures may play a role in its patterning the migrating IM (see below).

The Pronephric Intermediate Mesoderm Is Specified by Stage 6

An embryonic tissue is considered "specified" if it has been sufficiently patterned such that it will differentiate if placed in a "neutral" environment (Slack, 1991). In order to determine the state of specification of the avian IM, prospective IM from various stages of development (as determined from the fate map, Fig. 3) was dissected from chick embryos and grown in tissue culture in serum-free medium. As shown in Fig. 4, prospective IM from as early as stage 6 expressed the kidney markers pax-2 (100%, $n = 12$) and lim-1 (83%, $n = 6$) robustly when placed in culture.

In these experiments, the prospective IM was cultured with its attached ectoderm. It was found that, if the ectoderm was removed, the cultures exhibited a significant amount of cell death, although in many cases they still expressed kidney markers. In order to determine whether the ectoderm was playing a specific role in inducing IM gene expression, the ectoderm was removed and replaced with ectoderm from either lateral or anterior regions of the embryo. It was found that these other sources of ectoderm also promote cell survival and robust IM gene expression (Figs. 4C and 4D; lateral plate ectoderm 90% pax-2-positive, $n = 10$; anterior ectoderm, 100% pax-2-positive, $n = 3$), implying that ectoderm is required for IM survival in culture but that it does not play a specific role in inducing IM gene expression. Other studies have found that the ectoderm is required for high level expression of pax-2 and lim-1 in the nephric duct *in vivo* (Obara-Ishihara *et al.*, 1999).

Previous reports have found that somitic tissue plays a role in patterning the IM (Seufert *et al.*, 1999; Mauch *et al.*, 2000). When cultures were examined for the expression of the somite marker paraxis (Burgess *et al.*, 1995), they were found to be negative (Fig. 4F), demonstrating that the explanted IM did not contain patterned somite tissue and implying that the prospective IM that was used in these experiments did not require somite tissue to differentiate in culture. As a negative control, lateral plate mesectoderm from several stages was never found to express pax-2 (for example, see Fig. 4E) (stage 6, 0% pax-2-positive, $n = 6$; stage 7, 0%, $n = 8$; and stage 8, 0%, $n = 6$).

These experiments demonstrate that, shortly after emerging from the primitive streak, the prospective pronephric IM is already specified to express IM genes. Indeed, it is possible that the IM is specified even earlier. However, prior to gastrulation, the germ layers and tissue primordia are not well demarcated (Table 1; Psychoyos and Stern, 1996). Therefore, it is not feasible to culture prospective IM in the absence of other tissues which might be playing patterning roles.

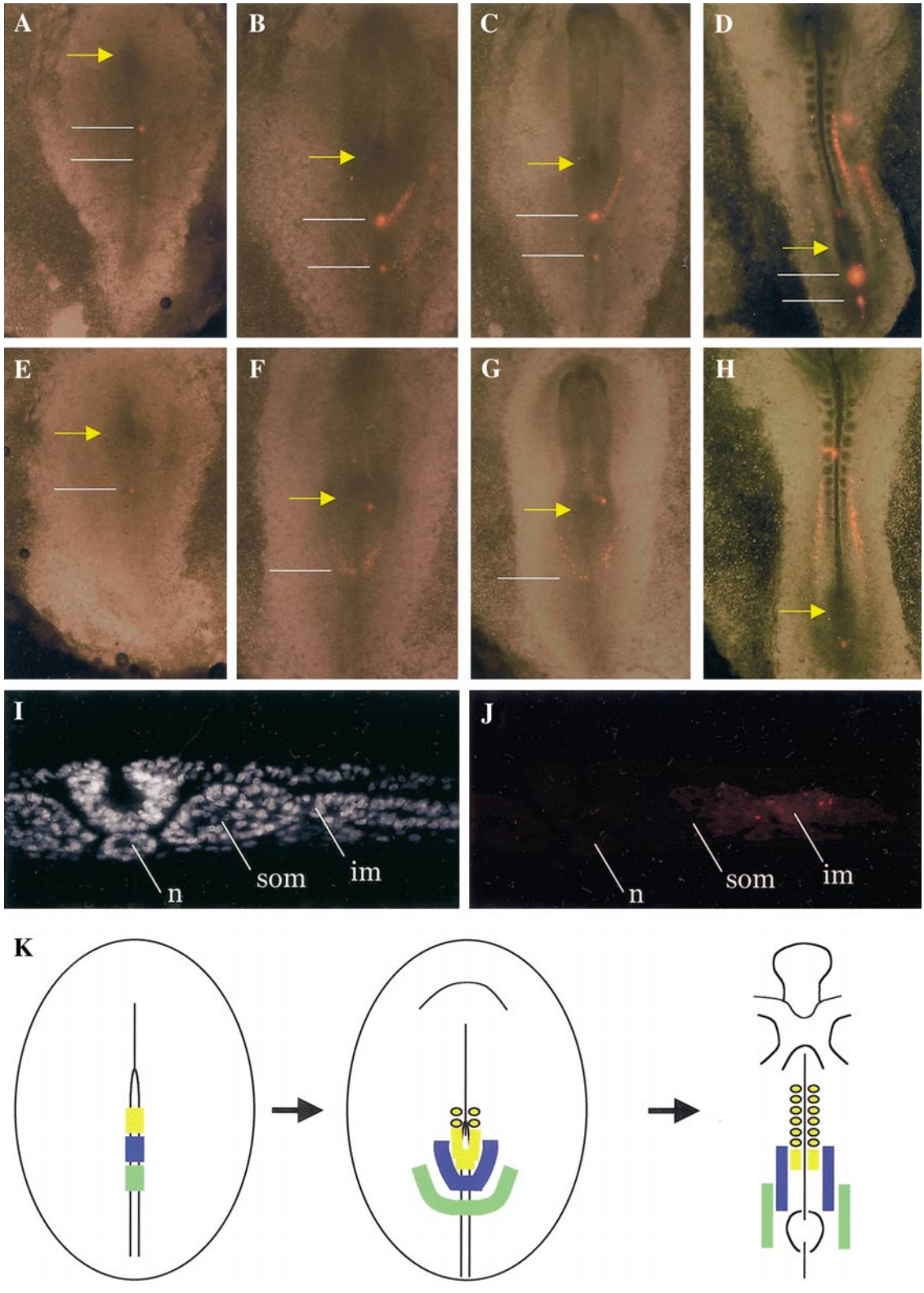
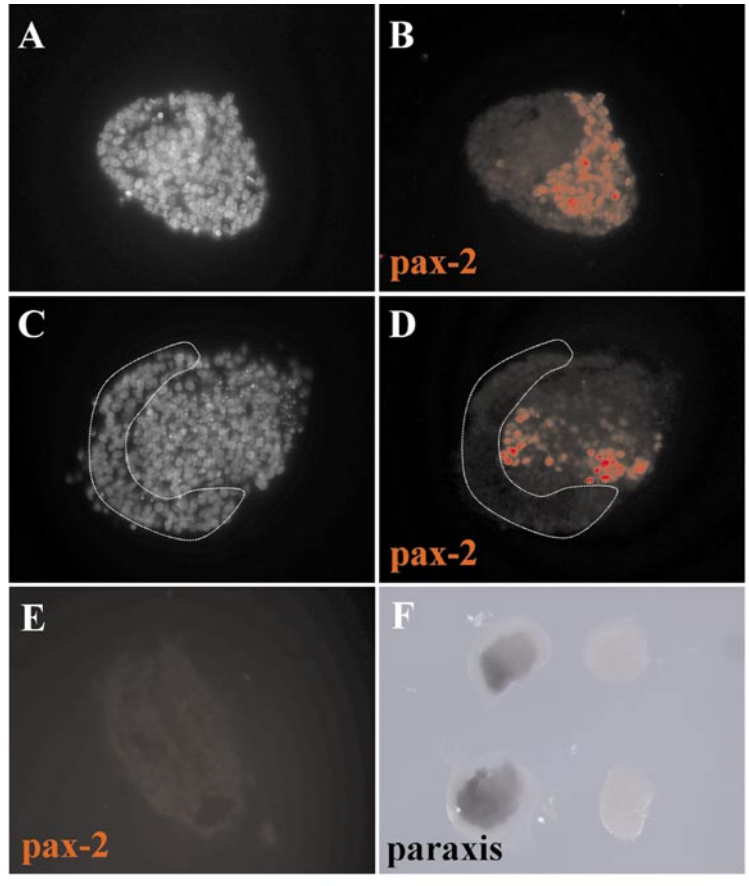


FIG. 3. Fate map of the chick pronephric region. Embryos were injected with DiI at stage 4-5 with the fluorescent lipophilic dye DiI (A, E) and were photographed at stage 6 (B, E), stage 7 (C, G), and stage 10 (D, H). White lines indicate sites of injection. Yellow arrows indicate the position of Hensen's node. The embryo in (A–D) was injected in two locations, one of which gave rise to cells in the IM and lateral somite and the other to cells of the LP. The embryo in (E–H) was injected in one location, which gave rise to cells located in the IM. (I, J) Section of a representative embryo stained with DAPI to show nuclei (I) and viewed under rhodamine optics to visualize DiI (J). In this embryo, DiI is found in the IM as well as the lateral somite and medial lateral plate. (K) Summary of fate map data, showing migration paths of prospective somite (yellow), intermediate mesoderm (blue), and lateral plate (green) at stage 5 (left), stage 7 (middle), and stage 9 (right). im, intermediate mesoderm; n, notochord; som, somite.



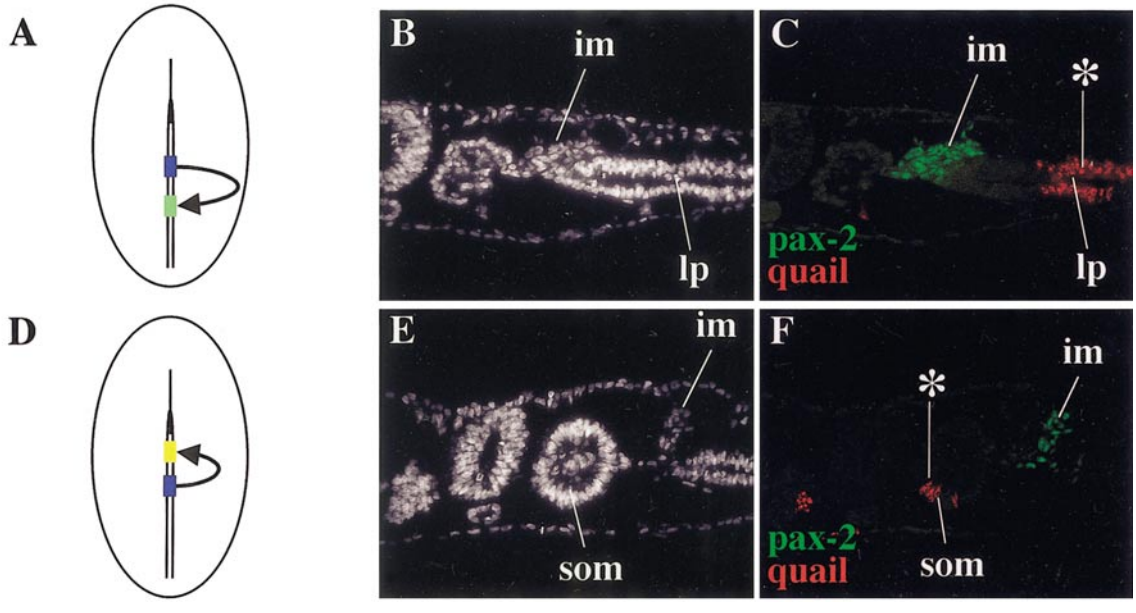


FIG. 4. Pronephric intermediate mesoderm is specified by stage 6. (A, B) Prospective IM and adjacent ectoderm from stage 6 embryos was placed in a defined culture medium. The explant expressed pax-2 (B). (C, D) Prospective IM from stage 6 embryos was cultured with ectoderm from the far lateral region of the embryo. The IM expressed pax-2 (D). The dotted line in (D) encloses the ectoderm. (A, C) DAPI staining of nuclei of explants. (E) Explant of lateral plate mesectoderm, which does not express pax-2. (F) Explants of stage 7 presomitic mesoectoderm (left) and prospective IM and adjacent ectoderm (right) analyzed for the expression of the somite marker paraxis. The control paraxial mesoderm expressed paraxis, while the prospective IM explants did not.

TABLE 3
Transplants between Intermediate Mesoderm and Adjacent Tissues

Donor quail	Host chick	% with Pax-2	% with many Pax-2	% with few Pax-2	Total *n*
St. 5 pre-IM	St. 5 pre-IM	100	100	0	2
	St. 5 pre-SOM	0	0	0	7
	St. 5 pre-LP	27	67	33	11
St. 5 pre-LP	St. 5 pre-IM	100	63	37	8
St. 5 pre-SOM	St. 5 pre-IM	77	90	10	13
St. 8 IM	St. 8 IM	100	100	0	4
	St. 5 pre-SOM	100	100 ($n = 6$)	0 ($n = 6$)	13
	St. 8 PSM	73	100 ($n = 5$)	0 ($n = 5$)	11
	St. 8 LP	29	0 ($n = 6$)	100 ($n = 6$)	14
St. 8 LP	St. 8 IM	62	100	0	13
St. 9 IM	St. 9 SOM	91	ND	ND	11
St. 9 IM	St. 9 LP	54	100	0	13

Note. Tissues were transplanted from quail donors into chick hosts, and the chimeras were examined for quail cells that expressed the kidney marker Pax-2. *Rows* indicate the operation. For example, the first row denotes an operation in which stage 5 quail tissue from the pre-IM region of the primitive streak was transplanted into the pre-IM region of a stage 5 chick primitive streak. All stage 5 transplants used primitive streak tissues, whereas transplants done after stage 5 used gastrulated mesodermal tissues. *Columns* indicate the percentage of grafts which contained pax-2-positive cells. A graft was counted as positive if it contained any pax-2-positive cells. Most of the positive grafts were then rescored as containing "many" or "few" Pax-2 cells. All sections from a given transplant that contained graft cells were examined. Transplants which contained at least one section that contained five or more pax-2-positive quail cells were counted as containing "many" pax-2 cells; transplants which contained pax-2-positive quail cells but in which no section contained more than five such cells were counted as containing "few" pax-2 cells; and transplants containing no pax-2-positive quail cells were counted as negative. Note that the numbers in columns 4 and 5 are given as the percentage of pax-2-positive transplants. psm, presegmental mesoderm. Other abbreviations as in Table 1.

Determination of the Intermediate Mesoderm

If an embryonic tissue is able to develop along its normal differentiation pathway when placed in an "antagonistic" environment which promotes other differentiation pathways, the tissue is considered "determined" with respect to that particular environment (Slack, 1991). Transplant and explant experiments were performed in order to study the determination of the IM. The goal of these studies was to elucidate how, and at which developmental stages, various embryonic tissues regulate the formation of the IM.

The Prospective Pronephros Is Not Determined When It Resides in the Primitive Streak at Stage 5

The first series of transplants were performed between anterior and posterior regions of the primitive streak. Donor tissue was taken from the prospective IM (600-800 microns from node) of stage 5 quail primitive streaks. Donor pieces were placed into the prospective lateral plate (Fig. 5A), prospective somite (Fig. 5D), or prospective IM (controls) regions of the primitive streak of recipient chicken embryos. These were grown for 24 h and assayed for the expression of pax-2 in quail cells. Control homotypic grafts (IM into IM) expressed pax-2 after culture and demonstrate that the grafting procedure does not affect IM gene expression (Table 3). In each of the other cases, prospective IM did not maintain its identity in the grafted locations. Only a small fraction of transplants into the posterior streak (27%, $n = 11$; Table 3; Figs. 5B and 5C) and no transplants into the anterior streak (0%, $n = 7$; Table 3; Figs. 5E and 5F) expressed pax-2. In all of the cases where pax-2 expression was seen in the lateral plate location, the graft was immediately adjacent to the endogenous IM. Thus, at stage 5, the IM is not determined to express Pax-2, and the grafted prospective IM presumably follows the patterning cues present in the environment into which it is placed.

Like the prospective IM, the prospective LP is also relatively unpatterned when it resides in the primitive streak. Posterior primitive streak transplanted into the IM region of the streak at stage 5 expressed pax-2 in 100% of

FIG. 5. Prospective pronephric IM is not committed at stage 5, when it resides in the primitive streak. Quail prospective IM (blue square in A, D) was transplanted to the prospective lateral plate region (green square in A) or the prospective somite region (yellow square in D) of chick embryos. Embryos were grown to stage 10-11 and stained with DAPI to mark nuclei (B, E) and with antibodies to quail cells (red, C, F) and to pax-2 (green, C, F). Transplanted quail cells did not express pax-2 and incorporated into the lateral plate (C) and somite (F). im, intermediate mesoderm; lp, lateral plate; som, somite. Asterisk (*) indicates grafted cells.

cases (n = 8; Table 3). However, transplants of prospective somitic streak to the prospective IM streak location gave somewhat different results. First, 32% (n = 19) of grafts into the 600-800 micron location were located in the somitic region after incubation. These embryos had an endogenous IM that was located the normal distance from the midline and exhibited normal morphology (data not shown). Of the other grafts, 77% (n = 10) expressed pax-2 (Table 3) and 54% appeared to form ectopic somite structures. Often, pax-2 expression and ectopic somite structures occurred within the same graft at different axial levels. These experiments demonstrate that, while the IM and LP are relatively unpatterned at primitive streak stages, presomitic mesoderm exhibits a somewhat greater degree of commitment at this time.

Stage 8- IM Is Determined with Respect to Axial Patterning Signals

In order to determine when prospective IM becomes determined with respect to axial patterning signals, stage 7-8 prospective IM was isochronically transplanted into the prospective somite region. As shown in Figs. 6A–6C and Table 3, most stage 8 IM grafts expressed pax-2 after transplantation to the paraxial region. These grafts remained as discrete entities and did not, in general, integrate into the nearby somite.

One possible explanation for this result is that the stage 8 paraxial region has different signaling properties than the stage 5 anterior primitive streak, which, in Figs. 5D–5F, was found to repattern the stage 5 prospective IM. In order to investigate this issue, stage 8$^-$ prospective IM was transplanted into the stage 5 anterior primitive streak. In these experiments, the grafted IM expressed pax-2 100% of the time (n = 13) (Table 3; Figs. 6D–6F), and indeed often appeared enlarged compared with the isochronic grafts of Figs. 6A–6C. None of the grafts displayed any suggestion of somite morphology. These results imply that, by stage 8$^-$, the IM has become determined with respect to axial/paraxial patterning cues, and that at this stage axial tissues might positively regulate IM formation.

Lateral Plate Contains an Activity That Inhibits IM Gene Expression

Although stage 8$^-$ IM appears determined with respect to axial patterning cues (Figs. 6A–6F), or when explanted in culture (Fig. 4), it was still important to test whether it could be influenced by other trunk mesodermal environments. Therefore, stage 8 quail intermediate mesoderm was transplanted into chick lateral plate (Fig. 6G). After growth overnight, IM grafts expressed Pax-2 at a much lower frequency in the lateral plate environment (29%, n = 14; Table 3; Figs. 6H and 6I) than in the somitic or node environments (Table 3; Figs. 6A–6F). Typically, grafts became integrated into the lateral plate (Figs. 6G–6I). To quantify differences seen between stage 8 lateral plate and somitic grafts, the number of pax-2-positive cells observed in each section of every graft was counted, and grafts were classified as having many, few, or no pax-2 cells (see Table 3 for definition of categories). "All grafts of IM into stage 8 PSM and stage 5 anterior primitive streak that were counted as pax-2 positive contained many pax-2-expressing cells, as did control grafts into the IM itself (Table 3). In contrast, all IM grafts into the lateral plate that were pax-2 positive contained only a few pax-2-expressing cells." These results indicate that the lateral plate environment is not permissive for IM development through stage 8. By stage 9 (when pax-2 begins to be expressed in the IM, Figs. 2B and 2C), the IM becomes refractory to the inhibitory environment of the lateral plate: grafts of stage 9 IM into the stage 9 lateral plate were observed to express pax-2 54% of the time (n = 13) (Figs. 6J–6L; Table 3). Similar results were seen when lim-1 expression was used as a marker of IM gene expression in the types of transplants described in Fig. 6 (data not shown).

Axial Tissues, but Not Somitic Mesoderm, Promote IM Formation

The above experiments found that, while the lateral plate is repressive for IM formation, the paraxial region is permissive. In addition, Figs. 6D–6F found that stage 8 IM was apparently expanded when it was transplanted near Hensen's node. These results raised the question of whether axial tissues actively promote IM formation, and a series of experiments was performed in order to address this issue.

Initial experiments found that the lateral plate is competent to express IM genes when transplanted into the IM as late as stage 8 (Figs. 7G and 7H). Therefore, lateral plate was used as a responder tissue in this set of experiments. Grafts of several types of quail axial tissue were placed into the lateral plates of stage 7-8 chick embryos at the same axial level as Hensen's node in order to test the IM-promoting activity of the axial tissues. The location and time of transplantation were chosen because, at this stage, the IM is still subject to negative regulation by the lateral plate and is determined relative to axial tissues (Fig. 6). Therefore, any positive effects on IM gene expression in these experiments could reveal potential interactions between axial and lateral patterning cues.

After analyzing many experiments, it appeared that axial tissues induced three types of response in the host (Table 4), which are defined first and then discussed in greater detail below. One response was induction of *ectopic* pax-2, which was defined as additional pax-2 that was not continuous with the endogenous Pax-2 expression pattern (Figs. 7A and 7B, asterisks). A second category was *expansion* of the IM, which was defined as additional pax-2 expression that was continuous with the endogenous IM (Fig. 7A, white arrow). The third category was *morphological changes*, which were defined as abnormalities in pax-2 expression that were correlated with decreases in expression elsewhere, and thus could reasonably be attributed to alterations in IM morphology (Figs. 7C and 7D, arrow). In order to distinguish between "expansion" or "ectopic" on the one hand vs

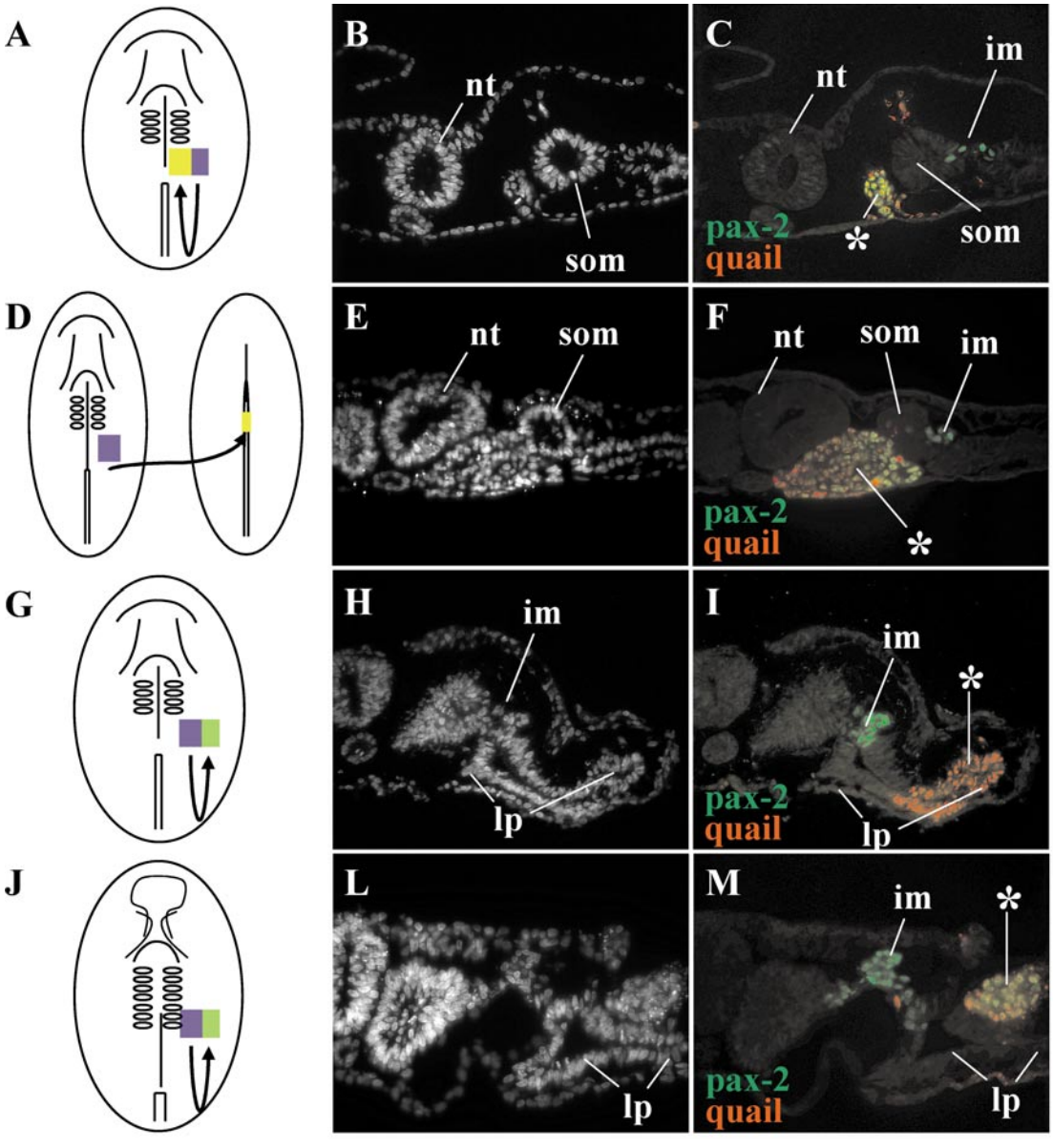


FIG. 6. State of commitment of IM at stages 8-9 with respect to transplantation into the somite and lateral plate regions. (A–C) Prospective IM (blue square in A) from stage 8 quail embryos was transplanted into the prospective somite region of stage 8 chick embryos (yellow square in A). Embryos were grown to stage 10-11 and stained with DAPI to mark nuclei (B) and with antibodies to quail cells (red, C) and to pax-2 (green, C). Transplanted quail cells expressed pax-2. (D–F) As in (A–C), but host was the somite-forming region of a stage 5 quail embryo (yellow box in D). Transplanted quail cells expressed pax-2 robustly. (G–I) As in (A–C), but the host was the lateral plate of a stage 8 quail embryo (green box in G). The grafted quail cells did not express pax-2. (J–M) The donor was quail stage 9 IM, and the recipient was chick stage 9 lateral plate. The grafted cells expressed pax-2 robustly. im, intermediate mesoderm; lp, lateral plate; nt, neural tube; som, somite. Asterisk (*) marks position of the grafts.

"morphological change" on the other hand, we compared pax-2 gene expression on the grafted vs the control side. "Morphological change" can appear at first glance to look like "ectopic" (Fig. 7D) because there is a region of Pax-2 expression that is discontinuous with the rest of the IM on that side. However, comparison of the two sides reveals that the anterior/posterior and medio/lateral extent of pax-2 expression is not altered on the grafted side in Fig. 7D (compare with Fig. 7B), and thus the alteration of pax-2 expression in Fig. 7D could be attributed to bunching of IM around the graft, with a concomitant loss of IM from the immediately posterior region.

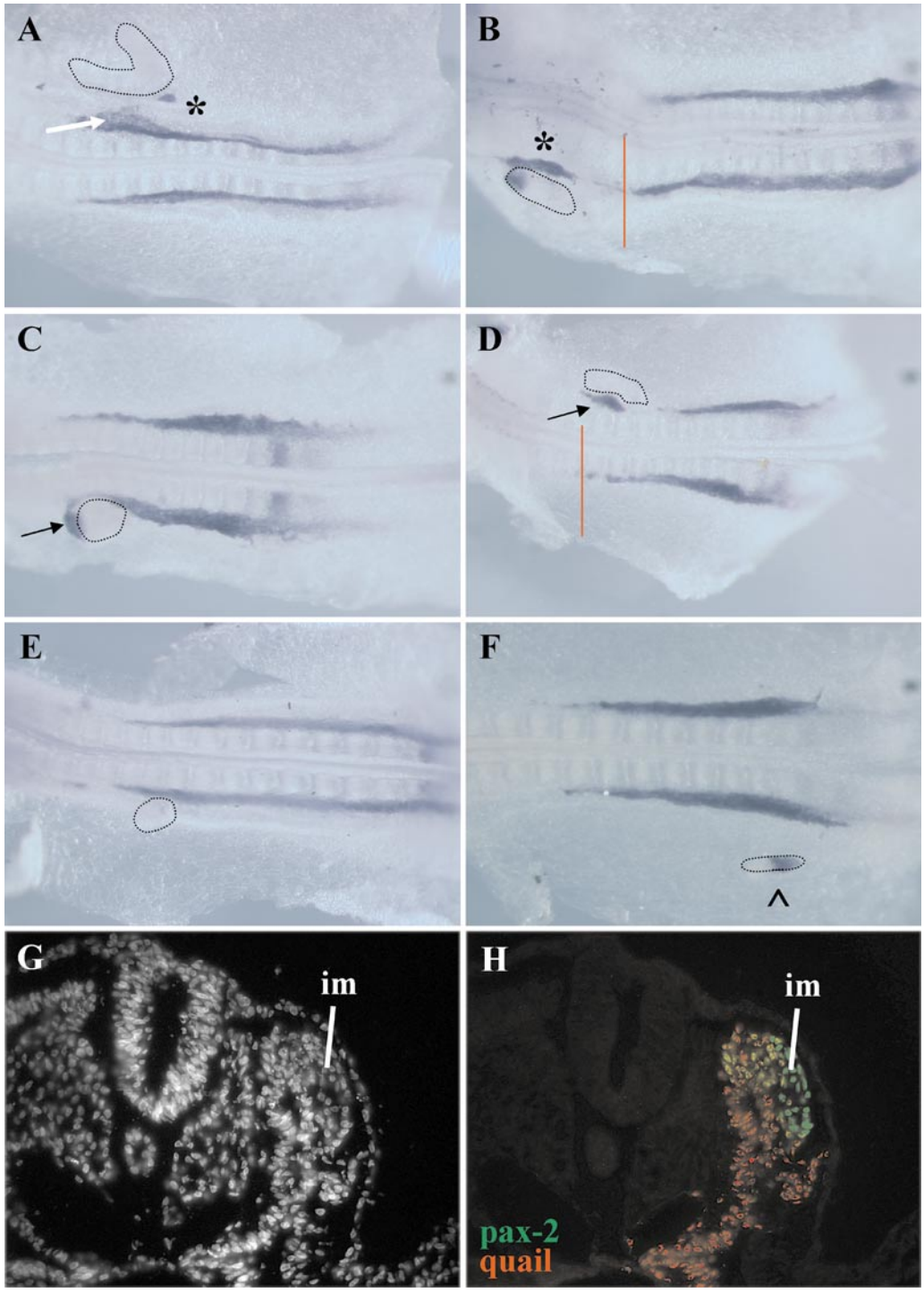


FIG. 7. Effects of axial tissues on patterning of the IM. Stage 5 Hensen's node (A, B), stage 8 presomitic mesoderm plus neural plate and notochord (C, D), or stage 8 somite alone (E, F) were grafted into the lateral plate of stage 7-8 embryos. The embryos were grown to stage 12-13 and analyzed for expression of pax-2 by *in situ* hybridization. Hensen's node induced ectopic expression of pax-2 (asterisk in A, B) as well as expansion of the endogenous IM (white arrow in A). In some node grafts, pax-2 was also expressed in the graft itself (B). Grafts of somite plus neural tube and notochord (C, D) primarily induced morphological changes in the endogenous host IM, such as wrapping of the endogenous IM around the graft (arrow in C) or bunching of the IM around the graft with an accompanying gap in the remaining IM (arrow in D). None of these grafts expressed pax-2. Grafts of somite alone (E, F) produced little alteration in IM gene expression patterns, but often expressed pax-2 in the graft (caret in F). (G, H) Stage 8 lateral plate is competent to express IM genes. Stage 8 quail lateral plate mesoderm was grafted into the IM of a stage 8 chick embryo. A portion of the graft was induced to express pax-2 (H).

TABLE 4
Effects on IM Patterning upon Grafting of Axial Structures into the Lateral Plate

	Ectopic IM	Expansion of IM	Morphological changes	No change	*n*
Node	26	63	16	21	19
SOM/NP/NC	0	18	53	29	17
PS/SOM	0	10	5	85	20

Note. The quail tissue listed in each *Row* was transplanted into the lateral plate of stage 7–8 chick embryos. Chimeras were examined for the effect on Pax-2 expression in the host chick tissue. "Ectopic IM" was defined as additional pax-2 that was not continuous with the endogenous Pax-2 expression domain. "Expansion of IM" was defined as additional pax-2 expression at the axial level of the graft that was continuous with the endogenous IM. "Morphological changes" were defined as abnormalities in pax-2 expression at the axial level of the graft that were correlated with decreases in expression elsewhere, and thus could reasonably be attributed to alterations in IM morphology or migration. See text for further discussion of the categories. The table entries give the percentage of grafts with the indicated effect, with the final column giving the number of embryos examined. nc, notochord; node, Hensen's node; np, neural plate; ps, presegmental mesoderm; som, somite.

With these definitions in mind, we can now describe the effects of the various grafted tissues. At stage 7-8, the only organized axial structure near the prospective pronephric IM is Hensen's node (see fate map, Figs. 3C and 3G). Grafts of stage 5–6 Hensen's node into the lateral plate induced ectopic foci of pax-2 gene expression in several cases (Figs. 7A and 7B, asterisks; Table 4). Sometimes, ectopic expression was seen lateral to the endogenous IM (Fig. 7A) and in other cases significantly anterior (Fig. 7B). Additionally, many experiments resulted in visible expansion of the endogenous IM into the lateral plate mesoderm (Fig. 7A, arrow; Table 4). It should be noted that Hensen's node was sufficient to promote IM expansion into lateral plate even if it was grafted at distances greater than one somites' width from the endogenous IM (Fig. 7A). However, ectopic pax-2 was never seen in locations lateral to the graft

As Hensen's node is a complex structure that gives rise to or can induce neural plate, somite, and notochord, any or all of these tissues might be responsible for the results seen in the node grafts. To explore this issue, grafts containing stage 8-8$^+$ somite–neural plate–notochord were placed into the stage 7-8 lateral plate (Figs. 7C and 7D; Table 4). The results of these experiments were quite different from those of the node transplants. The primary response appeared to be morphological changes induced in the endogenous IM. These axial/paraxial grafts seemed to attract the developing pronephric tissue, with the result that host pronephric tissue was often seen wrapped around the graft (Fig. 7C, arrow). In several examples, pax-2-expressing tissue accumulated around the graft, resulting in a diminution of the posterior IM (Fig. 7D, arrow). Unlike the results with Hensen's node grafts, no effect of the axial/paraxial grafts on pax-2 expression was seen when the graft was not immediately adjacent to the endogenous IM.

Finally, grafts of somite or presomitic mesoderm alone were performed. These rarely had any effect on IM development (Figs. 7E and 7F; Table 4). Interestingly, pax-2 expression was seen frequently (10/20) within grafted somites (Fig. 7F, caret), but not in grafts of somite plus neural plate and notochord, suggesting that somites might lose patterning in the absence of neural plate–notochord.

Taken together, these data suggest that the Hensen's node exhibits unique IM-promoting activities that are not recapitulated by other axial/paraxial tissues.

Repatterning of Paraxial Mesoderm to IM by the Lateral Plate

During analysis of the above experiments, it was observed that, when somites were grafted into the lateral plate, parts of the graft often expressed pax-2 (Fig. 7F). It should be noted that most of the grafts maintained somite morphology, even if some part of the labeled tissue expressed pax-2. These results suggested that somitic tissue may be partially repatterned into IM by placement into the lateral plate. In order to investigate this observation further, somites were combined with lateral plate in tissue culture. Cultures of somitic (Figs. 8A and 8B; 0%, $n = 16$) or lateral plate (Fig. 4E) mesectoderm alone showed no pax-2 or lim-1 gene expression. When somitic mesoderm and lateral plate mesectoderm were combined, pax-2 and lim-1 were strongly induced in the somitic tissue (Figs. 8C and 8D; 80% pax-2-positive, $n = 5$) Significantly, in these experiments, the somite did not induce IM gene expression in the lateral plate (0% pax-2-positive, $n = 5$). These experiments show that the lateral plate can reprogram paraxial tissues to form IM and that the paraxial tissues can respond to these signals as late as stage 7-8.

DISCUSSION

Summary of Pronephric IM Development

If one integrates the data from these studies and those of other studies, the following picture emerges of the patterning of the pronephric intermediate mesoderm. While still in the primitive streak, the prospective IM is still relatively uncommitted and can be reprogrammed into somite or lateral plate tissue if transplanted (Fig. 5). Shortly after gastrulation (by stage 6, if not earlier), the prospective IM has attained a certain degree of specification and will express IM genes if placed into culture (Fig. 4). Shortly thereafter (by stage 7-8), the prospective IM has attained a higher degree of determination and can now no longer be reprogrammed to a somite fate by placement into a somite-inducing environment (Figs. 6A–6F). At this time, the prospective IM is still sensitive to signals from Hensen's node and axial/paraxial structures, which can promote IM formation (Figs. 6D–6F), and lateral signals, which repress it

(Figs. 6G–6I). As development proceeds, Hensen's node recedes and the neural plate folds into the neural tube, distancing these structures from the forming IM. By stage 9, when the expression of transcription factors such as lim-1 and pax-2 is initiated, the pronephric IM is in contact only with the somites medially, which do not have IM-promoting activity (Figs. 7E and 7F), and the IM is also resistant to inhibitory signals from the lateral plate (Figs. 6J–6M). It is therefore essentially patterned by this stage.

Based on these results and those of others (Schoenwolf *et al.*, 1992; Brennan *et al.*, 1998; Seufert *et al.*, 1999; Mauch *et al.*, 2000), we propose a two-step model for the patterning of the IM (Fig. 9). While tissue is still in the primitive streak, the prospective IM is relatively uncommitted. By stage 6, shortly after cells leave the primitive streak, a field of cells is generated which is specified to give rise to IM (Step 1). Although the explant experiments (Fig. 4) demonstrate the existence of Step 1 of the model, the data in the current study do not address the mechanism of this initial stage in IM patterning. Next, competing signals from the lateral plate and axial tissues modulate the number of cells that commit to an IM fate (Step 2).

The Lateral Plate and IM Patterning

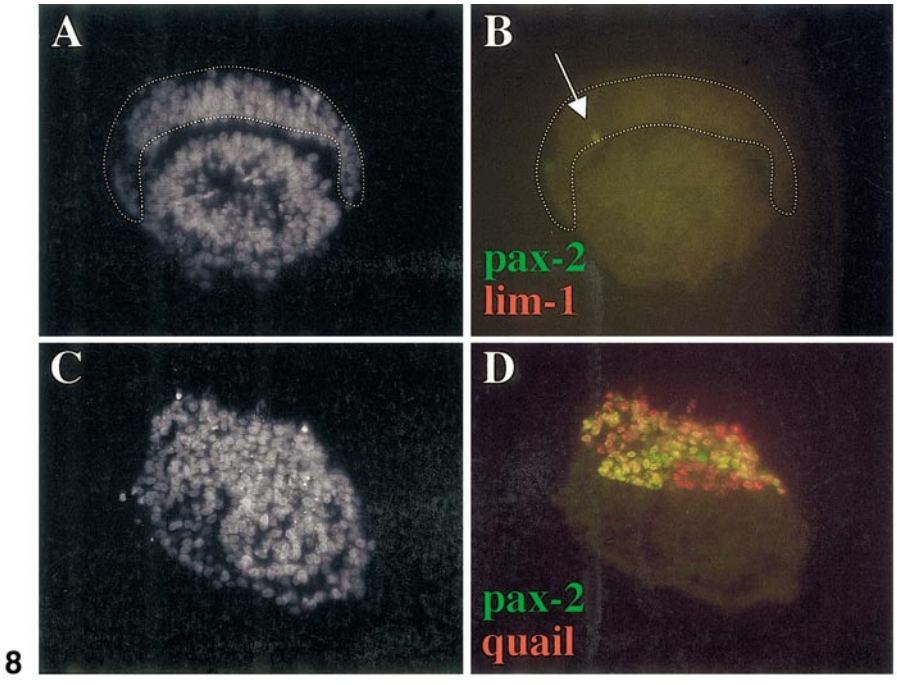
The existence of IM-inhibiting activity in the lateral plate is consistent with the findings of other studies. Misexpression of lim-1 and pax-2 in *Xenopus* embryos (Carroll and Vize, 1999) was found to lead to the formation of ectopic kidney tissue, but this ectopic kidney was only observed in the somite region and not in the lateral plate, which suggests that the lateral plate is nonpermissive for kidney differentiation, even if expressing lim-1 and pax-2. Additionally, experimental expansion of the somite to include the prospective IM region leads to repression of IM gene expression (Mariani *et al.*, 2001), rather than a displacement of IM tissue laterally. According to the proposed model, the somite–lateral plate border is now extended into a zone of higher IM-inhibiting activity, with the result that no IM tissue is formed. In the findings of Mauch *et al.* (2000), separation of the IM from axial tissues resulted in the inhibition of IM differentiation. In the current analysis, this operation, in which the IM remained attached to the lateral plate, would have changed the balance of signals to favor inhibition of IM formation.

The results of these studies indicate that the lateral plate contains an activity that can *repress* IM gene expression. The lateral plate also contains an activity which can *induce* IM gene expression in presomitic mesoderm (Figs. 7F, 8C, and 8D). While it is possible that these two effects could be mediated by two different activities in the lateral plate, one intriguing possibility is that both effects are mediated by the same signaling events. One model to explain the data would be the existence of a gradient of an activity along the mediolateral axis. The highest levels would promote lateral plate formation, intermediate levels would promote IM formation, and the lowest levels would promote somite formation. Transplantation of IM into LP would result in an increase of activity from medium to high and repatterning of the tissue as LP (Figs. 6G–6I), whereas transplantation of LP to the IM would result in a decrease of activity from high to medium and a repatterning of the LP into IM (Figs. 7G and 7H). Transplantation of somite into LP would lead to an increase in activity from low to medium and repatterning of the tissue to IM (Figs. 7F, 8C, and 8D). One obvious candidate for such an activity is signaling through the bone morphogenetic protein (Bmp) pathway. Bmp signaling is known to modulate dorsoventral mesodermal patterning in vertebrate embryos (Smith, 1995; Dosch *et al.*, 1997; Dale and Wardle, 1999). There is evidence that discrete levels of Bmp signaling can promote kidney tubule formation (Dosch *et al.*, 1997), although these experiments were performed at much earlier, pregastrulation stages of development. At later stages, Bmp signaling has been found to promote maintenance of high levels of kidney gene expression in the nephric duct and nephrogenic cord (Obara-Ishihara *et al.*, 1999). Studies are currently underway to test whether Bmp signaling mediates the repatterning events reported in these studies.

The Role of Axial Tissues in IM Patterning

Several previous studies have provided information regarding the role of axial tissues and, in particular, the somite in embryonic kidney formation. In some studies, axial/paraxial tissue has been found to be necessary and sufficient to promote kidney formation (Seufert *et al.*, 1999; Mauch *et al.*, 2000). On the other hand, in zebrafish embryos lacking Foxc (Topczewska *et al.*, 2001), or in *spadetail/one-eyed-pinhead* or *notail/one-eyed-pinhead* double mutants (K. Griffen, personal communication), early IM markers are expressed in the absence of somites. Also, in *Xenopus*, expansion of the somites into the lateral plate region does not result in kidney formation in the lateral plate (Mariani *et al.*, 2001).

The current studies are able to bring some clarity to these conflicting reports. We find that axial and paraxial tissues have a variety of effects on the developing IM, depending on the precise nature of the axial/paraxial tissue and the developmental stage of the IM. As assayed by their effects when grafted into the lateral plate, Hensen's node is able to induce ectopic IM gene expression and expansion of the IM; presomitic mesoderm with neural plate and notochord produces mostly morphological change in the endogenous IM; while somites or presomitic mesoderm alone do not significantly affect IM gene expression. It is significant that, at stage 7-8, when the transplant data show that Hensen's node can promote IM formation (Figs. 7A and 7B), the prospective IM is located lateral to the node (see fate map, Figs. 3C and 3G), supporting the hypothesis that the node actually participates in patterning the IM *in vivo*. When Hensen's node was combined with lateral plate in culture, only mild pax-2 gene expression was induced in the lateral plate (data not shown), suggesting that the node may induce IM indirectly and that robust IM induction by the node may



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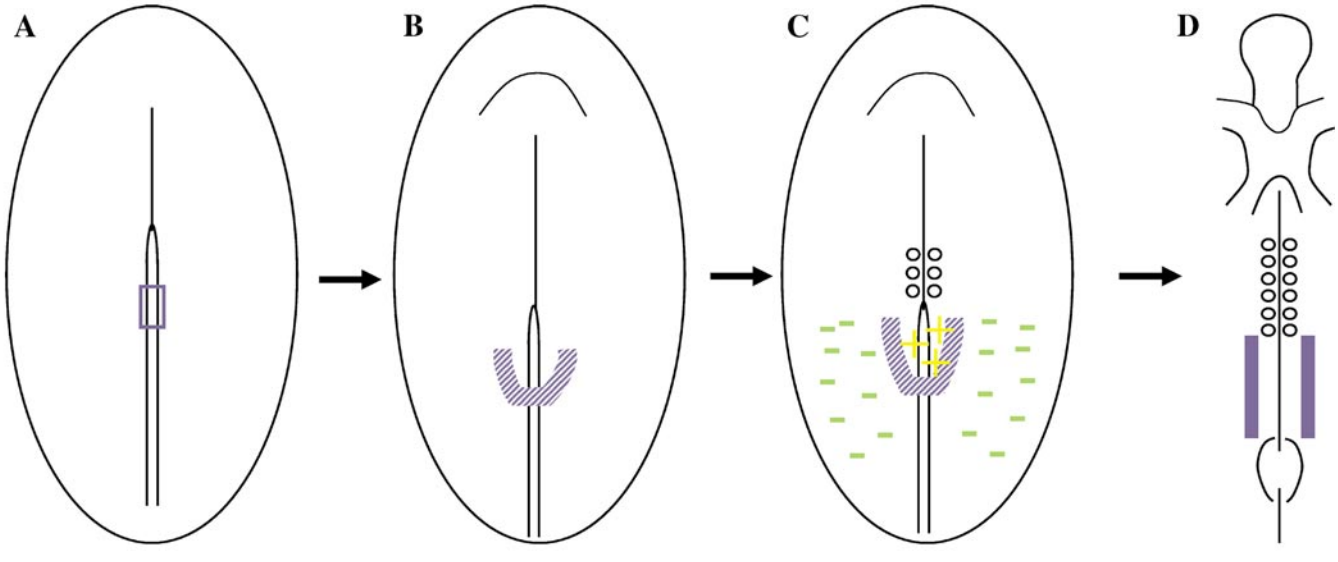

9 St. 5 St. 6 St. 8 St. 9

FIG. 8. Induction of IM from non-IM tissues at later stages. Stage 8 quail somite plus ectoderm (A, B) or quail somite together with chick lateral plate mesoectoderm (C, D) were cultured and analyzed for expression of lim-1 and pax-2 (B) or pax-2 and quail antigen (D). The somite alone did not express kidney markers (B), whereas somite did express pax-2 robustly when cultured with the lateral plate (D). In (B), the dotted line outlines the ectodermal portion of the explant. (A) and (C) are stained with DAPI to mark nuclei.

FIG. 9. Patterning of the pronephros. Based on data from this and other studies, a two-step model for pronephros patterning is proposed. When the prospective pronephros (blue box) resides in the primitive streak at stage 5, it is relatively uncommitted to an IM fate (A). By stage 6, shortly after migrating from the primitive streak, the prospective pronephros has become specified such that it will express kidney genes if placed into tissue culture (B). At stage 8 (C), the specified prospective IM is still subject to repression from signals in the lateral plate ("−" signs) and expansion from Hensen's node and perhaps other axial tissues ("+" signs). Between stages 8 and 9, the prospective pronephros attains a greater of commitment to a kidney fate, as it has become refractory to inhibitory lateral plate signals (D).

require a series of secondary interactions that are not replicated in culture.

The observation that IM is patterned by a balance between medial and lateral signals could help to explain one of the discrepancies between these results and those of a previous study, which found that somites could induce IM gene expression (Mauch *et al.*, 2000). The grafts in the current experiment may have been smaller than in the previous study. Somites were seen to induce IM expansion in a small percentage of the experiments in the current study (Table 4). It is possible that larger somite grafts might have a more pronounced effect.

All three of the axial/paraxial tissues examined in these studies can, with varying potencies, produce morphological changes in the pronephric rudiment. These axial/paraxial structures appeared to act as chemoattractants for the developing Wolffian duct, with the duct rudiment often found wrapped around the graft (Fig. 7C). In other cases, the graft appeared to attract IM cells locally, creating a break in the duct rudiment (Fig. 7D). Since the common denominator of these tissues was somite (Hensen's node induced somite formation in the host, and the other two grafts contained somite themselves), this effect may be mediated by the somite. Previous studies have found that, under a variety of experimental situations, the duct has a preference to grow alongside the somites (Holtfreter, 1944). The molecular basis for this preference is not known.

In considering the nature of the axial activity, it is also important to keep in mind that the nature of the response of the prospective IM changes over time. Prior to gastrulation, transplantation of prospective IM into the anterior primitive streak adjacent to Hensen's node promotes a somite fate in the prospective IM (Figs. 5D–5F). After gastrulation, grafts of IM into the same location induce, instead, an expansion of the IM (Figs. 6D–6F). The loss of competence to form somite tissue in response to axial signals may free the IM to respond to those same signals in other ways, thus allowing for the same signaling pathways to be used for different purposes at different stages of IM development.

Relationship between Pro-, Meso-, and Metanephric IM Patterning

Since the current studies focused on the formation of the pronephros, it is important to consider the relevance of these findings to patterning of the meso- and metanephric IM. The meso- and metanephric IM appear to share many of the patterning features of the pronephric IM that were identified in the current studies. The pro- and mesonephric IM (and likely the metanephric IM as well, although this was not directly examined in these studies) emerge from the same region of the primitive streak, but at different times (Fig. 3). Therefore, they have the same relationship to axial and lateral plate tissues, and it is reasonable to propose that the meso- and metanephric IM are patterned by these tissues in similar ways. In addition, all three levels of the kidney are significantly patterned prior to the expression of genes indicative of the onset of kidney differentiation, such as the transcription factors lim-1, pax-2, and WT-1. In the meso- and metanephros, the Wolffian duct is required for kidney gene expression, but the IM is the only tissue that can express kidney genes in response to inductive interactions with the duct (Lombard and Grobstein, 1969; Unsworth and Grobstein, 1970; Saxen, 1987). Thus, the meso- and metanephric IM are patterned prior to their interaction with the duct, and the duct simply serves to trigger differentiation in these already-patterned tissues. In the pronephros, the current studies have found that the IM is specified by stage 6, well before the expression of known markers of kidney differentiation (Fig. 4).

One difference between the mechanisms of formation of the three levels of kidney tissue may be that the meso- and metanephros are arrested in an undifferentiated but specified state until the arrival of signals from the duct which trigger differentiation, whereas the duct component of the pronephros must be able to differentiate without inductive signals from the duct itself. Interestingly, we have found that recently gastrulated prospective mesonephric IM will express lim-1 and pax-2 strongly if placed in culture in the absence of duct (data not shown). So, the function of the duct with respect to the mesonephros may be to derepress an activity in the embryo that represses IM differentiation.

In summary, using the avian pronephros as a model system, these studies have found that formation of the intermediate mesoderm is regulated by a combination of lateral and axial signals. Future studies will be aimed at identifying the molecular mediators of these patterning events.

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